Construction of thiostrepton-inducible, high-copy-number expression vectors for use in Streptomyces spp.

Takano, Eriko; White, Janet; Thompson, Charles J.; Bibb, Mervyn J.

Published in:
Gene

DOI:
10.1016/0378-1119(95)00545-2

Gene, 166 (1995) 133–137

GENE 09259

Construction of thiostrepton-inducible, high-copy-number expression vectors for use in *Streptomyces* spp.

(*tipA*; flounder growth hormone; β-keto acyl synthase; *redD*; His_6 tag; *Streptomyces lividans*; *Streptomyces coelicolor*)

Eriko Takano[a], Janet White[a], Charles J. Thompson[b] and Mervyn J. Bibb[a]

[a]*John Innes Centre, Colney, Norwich NR4 7UH, UK; and* [b]*Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland*

Received by K.F. Chater: 18 April 1995; Revised/Accepted: 7 June 1995; Received at publishers: 8 August 1995

SUMMARY

A high-copy-number plasmid expression vector (pIJ6021) was constructed that contains a thiostrepton-inducible promoter, p_{tipA}, from *Streptomyces lividans* 66. The promoter and ribosome-binding site of *tipA* lie immediately upstream from a multiple cloning site (MCS) which begins with a *Nde*I site (5′-CATATG) that includes the *tipA* translational start codon (ATG), allowing the synthesis of native proteins. Transcriptional terminators occur just upstream from p_{tipA} and immediately downstream from the MCS. To demonstrate the utility of pIJ6021, two streptomycete genes and a growth hormone-encoding gene from flounder (*Paralichthys olivaceus*) were cloned in the vector and expressed in *S. lividans* or *S. coelicolor* A3(2). A derivative of pIJ6021, pIJ4123, has a unique *Nde*I site positioned downstream from a nucleotide sequence that encodes a His_6 sequence and thrombin cleavage site. pIJ4123 can be used to produce His-tagged fusion proteins that can be readily purified by Ni^{2+}-affinity chromatography; if necessary, the His_6 tag can be removed by digestion with thrombin. The vectors contain a kanamycin-resistance-encoding gene for the selection of transformants.

INTRODUCTION

Attempts to over-produce native and heterologous proteins in streptomycetes have relied largely on the use of hcn vectors with strong, constitutively expressed promoters (for reviews, see Engels and Koller, 1992; Anné and Van Mellaert, 1993; Molnár, 1994). For proteins that may be deleterious to cell growth, or where there may be a need to control the expression of a cloned gene for experimental purposes, a regulatable system is desirable. Transcription of the chromosomal *tipA* gene of *Streptomyces lividans* 66 (John Innes Centre strain 1326) is apparently induced at least 200-fold by low levels of the antibiotic thiostrepton (Th; Murakami et al., 1989); when cloned on a mcn plasmid in *S. ambofaciens*, transcription from the *tipA* promoter (p_{tipA}) appeared to be induced at least 60-fold upon addition of Th (Kuhstoss and Rao, 1991). *tipA* was cloned and characterised (Murakami et al., 1989) and the regulation of p_{tipA} studied in detail (Holmes et al., 1993). p_{tipA} has been used to regulate gene expression in integrative vectors (Smokvina et al., 1990), and in mcn and hcn plasmids (Murakami et al., 1989; Kuhstoss and Rao, 1991; Holt et al., 1992). Here, we describe the construction of two hcn, p_{tipA}-based

Correspondence to: Dr. M.J. Bibb, John Innes Centre, Colney, Norwich NR4 7UH, UK. Tel. (44-1603) 452-571; Fax (44-1603) 456-844; e-mail: bibb@bbsrc.ac.uk

Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); FGH, flounder growth hormone; hcn, high copy number; *kan*, gene encoding Km^R from *Micromonospora echinospora*; Km, kanamycin; kb, kilobase(s) or 1000 bp; mcn, moderate copy number; MCS, multiple cloning site(s); nt, nucleotide(s); ORF, open reading frame; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; p_{tipA}, *tipA* promoter; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; ^{R}, resistance/resistant; RBS, ribosome-binding site(s); *S.*, *Streptomyces*; SDS, sodium dodecyl sulfate; t_{fd}, transcriptional terminator from phage fd; t_o, transcriptional terminator from phage λ (Hayes and Szybalski, 1973); Th, thiostrepton; *tipA*, Th-inducible gene from *S. lividans* 66; *tsr*, gene encoding Th^R from *S. azureus*; [], denotes the plasmid-carrier state.

SSDI 0378-1119(95)00545-5

vectors, pIJ6021 and pIJ4123. pIJ4123 can be used to produce His-tagged fusion proteins that can be readily purified by Ni^{2+}-affinity chromatography (Hoffmann and Roeder, 1991, and references therein). To demonstrate the utility of pIJ6021, two streptomycete genes and a growth hormone gene from the flatfish *Paralichthys olivaceus* (commonly known as flounder) were expressed in *S. lividans* or *S. coelicolor* A3(2).

EXPERIMENTAL AND DISCUSSION

(a) Construction of pIJ6021

A 115-bp synthetic p_{tipA} fragment (corresponding to nt 4–119 of the published *tipA* sequence; Holmes et al., 1993) was made that contained a *Nde*I site (5′-CATATG) overlapping the *tipA* translational start codon (ATG). This fragment was inserted into a derivative of the hcn plasmid pIJ487 (50–100 copies per chromosome; T. Kieser, personal communication; Ward et al., 1986) to give pIJ6021 (Fig. 1). pIJ6021 contains a MCS to facilitate fragment insertion. The t_o transcriptional terminator of *oop* RNA from phage λ (Hayes and Szybalski, 1973; Zukowski and Miller, 1986) occurs upstream from p_{tipA} to prevent transcriptional read-through from the vector and to maximise the induction ratio upon addition of Th. A transcriptional terminator from phage fd (t_{fd}; Gentz et al., 1981; Ward et al., 1986) is located downstream from the MCS to prevent potentially deleterious transcription of vector sequences from p_{tipA}. A Km^R gene (*kan*) from *Micromonospora echinospora* (C. Nojiri, personal communication) allows selection of transformants, and the Th^R gene (*tsr*) from *S. azureus* provides resistance to Th upon induction of p_{tipA} with the antibiotic. To assess the utility of pIJ6021, three functionally diverse genes were inserted in the vector, and their expression levels assessed before and after induction of p_{tipA} with Th.

(b) Application of pIJ6021

The *tcmK* gene of *S. glaucescens* GLA.0 encodes a 45-kDa β-keto acyl synthase that plays an early role in the synthesis of the polyketide antibiotic tetracenomycin C (ORF1 of Bibb et al., 1989; Gramajo et al., 1991). A 1.7-kb *Nde*I-*tcmK*-*Hin*dIII fragment, with the *Nde*I site overlapping the start codon of *tcmK*, was inserted between the *Nde*I and *Hin*dIII sites of pIJ6021 to give pIJ6024. Coomassie blue staining following SDS-PAGE revealed a protein of approx. 50 kDa (TcmK migrates more slowly then expected upon SDS-PAGE; Gramajo et al., 1991) in induced *S. lividans* 1326[pIJ6024] that was not apparent in uninduced cultures (Fig. 2A, upper panel). Western analysis using Ab raised to a synthetic oligopeptide corresponding to an internal segment of TcmK (Gramajo et al., 1991) confirmed that the 50-kDa protein was TcmK (Fig. 2A, lower panel), and also revealed lower amounts of the protein in uninduced *S. lividans* 1326[pIJ6024]; a corresponding band was not detected in *S. lividans* 1326[pIJ6021] grown with and without Th (Fig. 2A, lower panel).

Mature FGH is a 20-kDa protein derived from the primary translation product by removal of a 17-aa signal peptide that is normally required for secretion (Watahiki et al., 1989). The PCR (Erlich, 1989) was used to place a *Nde*I site at the beginning of the coding region of the mature FGH and a *Bam*HI site just beyond the 3′ end of the coding region. At the same time, codons 2 and 4 of the truncated *fgh* were changed so that the first 12 codons corresponded to synonymous codons that are used most frequently in streptomycete genes (Wright and Bibb, 1992) (expression of high-G+C streptomycete genes in *Escherichia coli* was enhanced by changing the first few N-terminal codons to the synonyms most frequently used in highly expressed *E. coli* genes; Gramajo et al., 1991; Angell et al., 1992). The 550-bp *Nde*I-*fgh*-*Bam*HI fragment was inserted in pIJ6021 that had been cleaved with *Nde*I + *Bam*HI to give pIJ6018. Coomassie blue staining following SDS-PAGE failed to reveal a protein of the expected size that was present only in induced cultures of *S. lividans* 1326[pIJ6018] (Fig. 2B, upper panel; the identity of the 27-kDa protein apparent only in these extracts is not known). However, Western analysis using Ab raised to FGH identified a protein of approx. 20 kDa that co-migrated with FGH and that was absent from uninduced cultures of *S. lividans* 1326[pIJ6018], and from extracts of *S. lividans* 1326[pIJ6021] grown with and without Th (Fig. 2B, lower panel).

redD is the putative pathway-specific activator gene for the undecylprodigiosin biosynthetic pathway of *S. coelicolor* (Narva and Feitelson, 1990; Takano et al., 1992). pIJ6021 was cleaved with *Nde*I, 5′ extensions were filled in with PolIk, and the DNA was cut with *Bam*HI. The vector fragment was ligated with a 1.44-kb blunt-*redD*-*Bam*HI fragment to give pIJ4123 (Fig. 1), in which the ATG start codon of an N-terminally extended *redD* ORF was positioned just downstream from the *tipA* RBS. Coomassie blue-staining following SDS-PAGE of the soluble fraction and pellet obtained after sonication of *S. coelicolor* JF1[pIJ4123] that had been induced with Th, and of total protein extracts of the same culture, revealed a protein of approx. 32 kDa that was absent from extracts of uninduced cultures (Fig. 2C, upper panel). Western analysis using Ab raised to RedD (J.W., unpublished results) confirmed that the 32-kDa protein was RedD (Fig. 2C, lower panel); no such signal was detected in extracts of *S. coelicolor* JF1[pIJ4123] grown without Th. A significant proportion of the RedD protein made in induced cultures appeared to be insoluble (Fig. 2C, upper panel, lanes S and P).

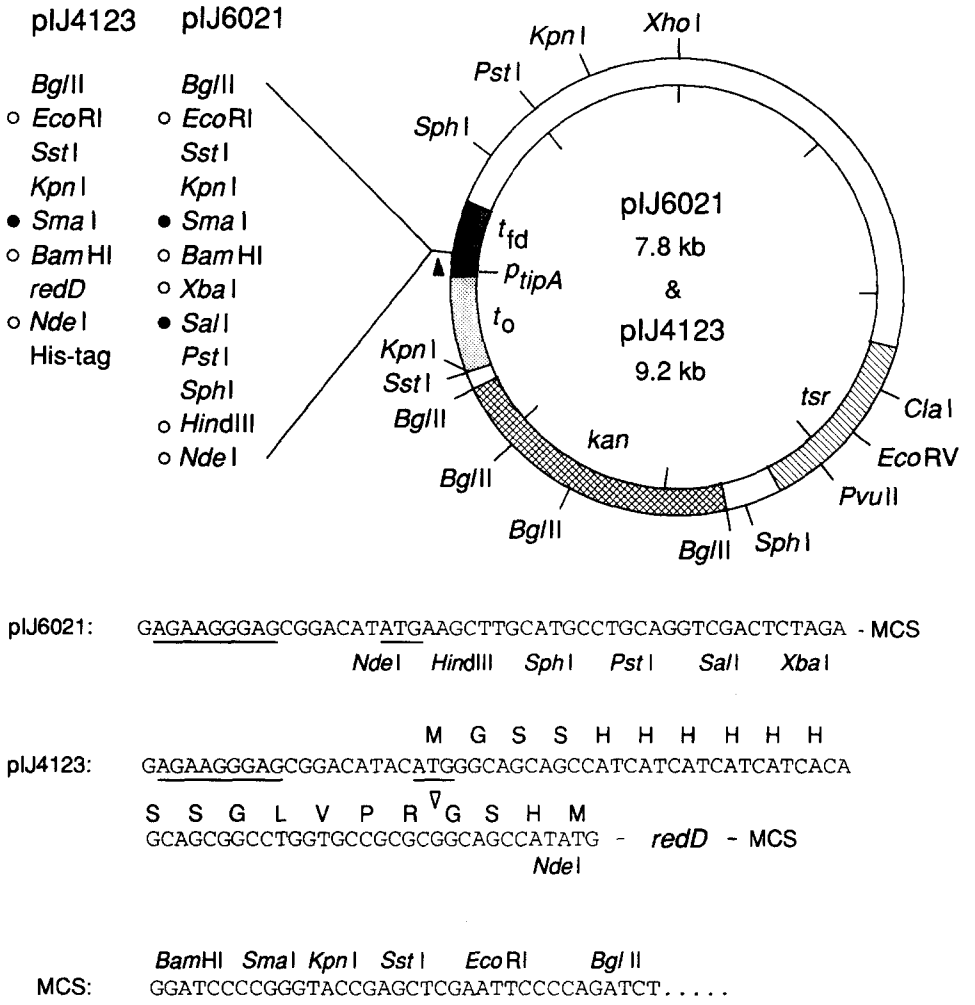

Fig. 1. Restriction maps of plasmids pIJ6021 and pIJ4123. Open circles indicate unique cloning sites present in the MCS; closed circles indicate that additional unmarked sites occur in the vectors; *redD* of pIJ4123 contains unmarked *Cla*I and *Pvu*II sites (one of each). The filled arrowhead indicates the direction of transcription from p_{tipA}. The *tipA* RBS and ATG start codon are underlined, and the open arrowhead indicates the site of cleavage by thrombin. The vectors were constructed using standard cloning procedures (Hopwood et al., 1985; Sambrook et al., 1989). Details of their constructions are available from the authors on request.

(c) pIJ4123 as a vector for the production of His_6-tagged proteins

The RedD protein produced from pIJ4123 has an N-terminal extension derived from the 65-bp *Nco*I-*Nde*I segment of pET15b (Novagen, Madison, WI, USA). The presence of a His_6 tag within this extension greatly aided the subsequent purification of the RedD fusion protein by Ni^{2+}-affinity chromatography (J.W., unpublished results). To take advantage of this facility, pIJ4123 can be cleaved with *Nde*I, or *Nde*I plus either *Bam*HI or *Eco*RI, to generate a linearised replicon containing p_{tipA} upstream from the sequence encoding the His_6 tag, which terminates at the unique *Nde*I site. A gene of interest with a *Nde*I site positioned to allow the production of a His-tagged fusion protein can then be inserted into the replicon, and the fusion protein readily purified by Ni^{2+}-affinity chromatography. Transformants of *S. lividans* or *S. coelicolor* containing uncut or religated vector produce enough RedD even in the absence of Th induction to yield intensely red colonies, providing a facile visual screen for recombinant clones.

(d) Concluding remarks

pIJ6021 has proved effective in expressing three functionally diverse genes in *S. lividans* or *S. coelicolor*: a eukaryotic growth hormone (*fgh*), a putative transcriptional activator (*redD*) and a membrane-associated β-keto acyl synthase (*tcmK*). It has also been used to express the *whiB* (E. Vijgenboom, personal communication) and *act*II-ORF4 (F. Malpartida, personal communication) genes of *S. coelicolor* in *S. lividans*. While the level of *fgh* expression was too low for detection by Coomassie blue staining, TcmK and RedD appeared to be made at levels of a few percent of total cell protein. Unlike TcmK, FGH and RedD were not detected by Western analysis prior to induction of p_{tipA} with Th. The inability to detect basal levels of *fgh* and *redD* expression might reflect the rapid

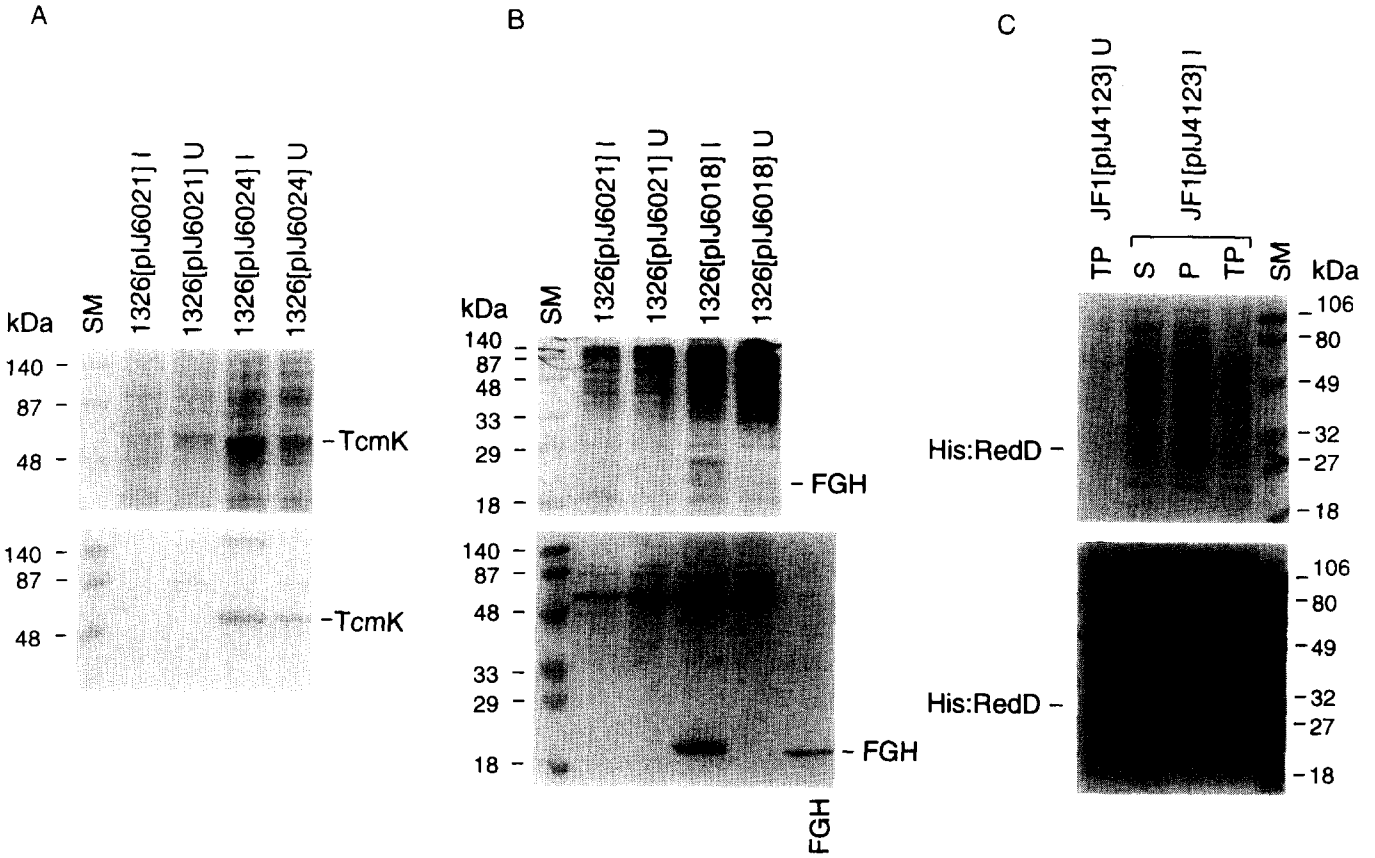

Fig. 2. Expression of native and foreign genes in pIJ6021. SM, size markers; U, uninduced; I, induced; TP, total protein; S, soluble fraction obtained after sonication; P, pellet obtained after sonication; FGH, 0.1 μg of purified FGH. (**A**) Expression of *tcmK* in pIJ6021. The 1.7-kb *Nde*I-*Hin*dIII fragment of pIJ2232 that contains *tcmK* (Gramajo et al., 1991) was ligated with the 6-kb *Nde*I-*Xho*I and 1.8-kb *Xho*I-*Hin*dIII fragments of pIJ6021 to give pIJ6024 (the adjacent locations of the *Nde*I and *Hin*dIII sites of pIJ6021 appeared to prevent digestion by both enzymes, precluding simple insertion of the *tcmK* fragment in *Nde*I + *Hin*dIII-digested pIJ6021). (**B**) Expression of *fgh* in pIJ6021. 20 pmol of the 5′ primer 5′-CATGGATCCCATATGCAGCCGATCACCGAGAACCAGCGCCTGTTGTCC and of the 3′ primer 5′-CATGGATCCGGTACTTCTTG-GCGGGACAGG, and 10 ng of a pBluescript KS(+) (Stratagene, La Jolla, CA, USA) derivative containing *fgh* were subjected to 35 cycles of the PCR (95°C for 30 s, 52°C for 30 s and 72°C for 1 min) in the presence of 10 mM Tris·HCl pH 8.3/2.5 mM $MgCl_2$/50 mM KCl/5% (v/v) glycerol/0.1% (w/v) gelatin/200 μm of each dNTP/2.5 units of *Taq* polymerase (Boehringer-Mannheim). The amplified fragment was gel-purified, cleaved with *Bam*HI, and inserted in the *Bam*HI site of pIJ2925 (Janssen and Bibb, 1993) to give pIJ6107; the sequence of the modified *fgh* was confirmed by double-strand sequencing using the Promega TaqTrack Sequencing System. pIJ6107 was digested with *Nde*I + *Bam*HI, the 550-bp fragment containing the modified *fgh* gel-purified and inserted in *Nde*I + *Bam*HI-digested pIJ6021 to give pIJ6018. (**C**) Expression of *redD* in pIJ6021. Synthetic oligodeoxy-ribonucleotides, extending from the start codon of *redD* to a *Cla*I site located 27 nt into the *redD* coding sequence (Takano et al., 1992), were used to create a *Nde*I site overlapping the *redD* start codon. A 1.38-kb *Nde*I-*redD-Bam*HI fragment was inserted in *Nde*I- and *Bam*HI-cleaved pET15b (Novagen) to give pIJ4116, which has a unique *Nco*I site located 59-bp upstream from the *Nde*I site. pIJ4116 was digested with *Nco*I, 5′ extensions filled in with PolIk, and the DNA cut with *Bam*HI to release a 1.44-kb blunt-*Nde*I-*redD-Bam*HI fragment. pIJ6021 was cleaved with *Nde*I, 5′ extensions filled in with PolIk, the DNA cut with *Bam*HI and ligated with the 1.44-kb fragment containing *redD* to give pIJ4123. *S. lividans* 1326 (Hopwood et al., 1985) or *S. coelicolor* strain JF1 (*argA1, guaA1, redD42, act*II-177, SCP1$^-$, SCP2$^-$; Feitelson and Hopwood, 1983) derivatives containing pIJ6021, pIJ6018, pIJ6024 or pIJ4123 were selected by flooding transformation plates with 1 ml of water containing 2 mg Km. After single colony purification and confirmation of the required construct by restriction analysis, derivatives were grown in 10 ml of YEME (Hopwood et al., 1985) containing 5 μg Km/ml in Universal (40 ml capacity) containers containing small stainless steel springs at 300 rpm at 30°C; transcription from p_{tipA} was induced after 12 h by addition of Th to a final concentration of 5 μg/ml. After a further 12 h of incubation, the cells were harvested by centrifugation. For the analysis of *fgh* and *tcmK* expression, samples of the mycelium were boiled in loading buffer (62.5 mM Tris·HCl pH 6.8/2% (w/v) SDS/10% (v/v) glycerol/5% (v/v) 2-mercaptoethanol/0.1% (w/v) bromophenol blue). For analysis of *redD* expression, mycelium was also disrupted by sonication, and samples of the soluble fraction and pellet boiled with equal volumes of 2 × loading buffer. Samples were fractionated by 0.1% SDS/12–14% (w/v) PAGE and protein bands visualised with Coomassie blue stain. Duplicate sets of samples were run on identical gels, transferred to nitrocellulose filters, and probed with 1:1000 dilutions of Ab raised in rabbits against FGH or a synthetic oligopeptide corresponding to TcmK (Gramajo et al., 1991), or with a 1:1000 dilution of Ab raised in a rat against RedD (J.W., unpublished data), as described by Burnette (1991). A goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bethesda Research Laboratories) was used as the second Ab. The top panels show the Coomassie-blue-stained gels, and the lower panels show the results of the Western analyses. The nature of the cross-reacting proteins of approx. 50 kDa observed in the lower panels of **B** and **C** is not known.

degradation of their fusion transcripts or protein products, or differences in the avidity of their cognate Ab. Although Western analysis failed to detect RedD in extracts of uninduced *S. coelicolor* A3(2) JF1[pIJ4123], the cultures produced large quantities of undecylprodigiosin, indicating that some RedD protein was made. Thus, genes cloned in these vectors appear to be expressed at basal levels even in the absence of Th induction. This may reflect transcription initiating at p_{tipA}, or elsewhere in the vector that is not completely terminated at t_o.

Induction of p_{tipA} requires *tipA* both in vitro (Holmes et al., 1993) and in vivo (T. Katoh and C.J.T., unpublished data). While *tipA* homologues apparently occur in many *Streptomyces* species, including the *S. lividans* and *S. coelicolor* strains used here, they are not present in all (E.T., unpublished data). Thus, to use these vectors in strains that lack *tipA*, the gene must be provided either by insertion in pIJ6021 or pIJ4123, or by introduction on a separate vector.

ACKNOWLEDGEMENTS

We thank Nippon Oil Company Ltd. for *fgh*, FGH and Ab to FGH, and for providing financial support to E.T., C. Nojiri for the *kan* gene from *M. echinospora*, and Mark Buttner, Keith Chater, David Hopwood and Gilles van Wezel for their comments on the manuscript. This work was supported in part by a grant-in-aid from the Biotechnology and Biological Sciences Research Council, and in part by grant BIOT-CT90-0255 from the European Union.

REFERENCES

Angell, S., Schwarz, E. and Bibb, M.J.: The glucose kinase gene of *Streptomyces coelicolor* A3(2): its nucleotide sequence, transcriptional analysis and role in glucose repression. Mol. Microbiol. 6 (1992) 2833–2844.

Anné, J. and Van Mellaert, L.: *Streptomyces lividans* as host for heterologous protein production. FEMS Microbiol. Lett. 114 (1993) 121–128.

Bibb, M.J., Biro, S., Motamedi, H., Collins, J.F. and Hutchinson, C.R.: Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI* genes provides key information about the enzymology of polyketide antibiotic biosynthesis. EMBO J. 8 (1989) 2727–2736.

Burnette, W.N.: Western blotting. Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. Anal. Biochem. 112 (1991) 195–212.

Engels, J.W. and Koller, K.P.: Gene expression and secretion of eukaryotic foreign proteins in *Streptomyces*. In: Murray, J.A.H. (Ed.), Transgenesis. Wiley, New York, NY, 1992, pp. 31–53.

Erlich, H.A.: PCR Technology. Stockton Press, New York, NY, 1989.

Feitelson, J.S. and Hopwood, D.A.: Cloning of a *Streptomyces* gene for an *O*-methyltransferase involved in antibiotic biosynthesis. Mol. Gen. Genet. 190 (1983) 394–398.

Gentz, R., Langner, A., Chang, A.C.Y., Cohen, S.N. and Bujard, H.: Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc. Natl. Acad. Sci. USA 78 (1981) 4936–4940.

Gramajo, H.C., White, J., Hutchinson, C.R. and Bibb, M.J.: Overproduction and localization of components of the polyketide synthase of *Streptomyces glaucescens* involved in the production of the antibiotic tetracenomycin C. J. Bacteriol. 173 (1991) 6475–6483.

Hayes, S. and Szybalski, W.: Control of short leftward transcripts from the immunity and *ori* regions in induced coliphage lambda. Mol. Gen. Genet. 126 (1973) 275–290.

Hoffmann, A. and Roeder, R.G.: Purification of his-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. Nucleic Acids Res. 19 (1991) 6337–6338.

Holmes, D.J., Caso, J.L. and Thompson, C.J.: Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. EMBO J. 12 (1993) 3183–3191.

Holt, T.G., Chang, C., Laurent-Winter, C., Murakami, T., Garrels, J.I., Davies, J.E. and Thompson, C.J.: Global changes in gene expression related to antibiotic synthesis in *Streptomyces hygroscopicus*. Mol. Microbiol. 6 (1992) 969–980.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H.: Genetic Manipulation of *Streptomyces*: A Laboratory Manual. John Innes Foundation, Norwich, 1985.

Janssen, G.R. and Bibb, M.J.: Derivatives of pUC18 that have *BglII* sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene 124 (1993) 133–134.

Kuhstoss, S. and Rao, R.N.: A thiostrepton-inducible expression vector for use in *Streptomyces* spp. Gene 103 (1991) 97–99.

Molnár, I.: Secretory production of homologous and heterologous proteins by recombinant *Streptomyces*: What has been accomplished? In: Murooka, Y. and Imanaka, T. (Eds.), Recombinant Microbes for Industrial and Agricultural Applications. Marcel Dekker, New York, NY, 1994, pp. 81–103.

Murakami, T., Holt, T.G. and Thompson, C.J.: Thiostrepton-induced gene expression in *Streptomyces lividans*. J. Bacteriol. 171 (1989) 1459–1466.

Narva, K.E. and Feitelson, J.S.: Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). J. Bacteriol. 172 (1990) 326–333.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Smokvina, T., Mazodier, P., Boccard, F., Thompson, C.J. and Guerineau, M.: Construction of a series of pSAM2-based integrative vectors for use in actinomycetes. Gene 94 (1990) 53–59.

Takano, E., Gramajo, H.C., Strauch, E., Andres, N., White, J. and Bibb, M.J.: Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 6 (1992) 2797–2804.

Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J., Buttner, M.J. and Bibb, M.J.: Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. Mol. Gen. Genet. 203 (1986) 468–478.

Watahiki, M., Yamamoto, M., Yamakawa, M., Tanaka, M. and Nakashima, K.: Conserved and unique amino acid residues in the domains of the growth hormones. J. Biol. Chem. 264 (1989) 312–316.

Wright, F. and Bibb, M.J.: Codon usage in the G+C-rich *Streptomyces* genome. Gene 113 (1992) 55–65.

Zukowski, M.M. and Miller, L.: Hyperproduction of an intracellular heterologous protein in a *sacU*^{h} mutant of *Bacillus subtilis*. Gene 46 (1986) 247–255.